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Analytical Methods

Response surface methodology for optimisation of protein concentrate preparation from cowpea [*Vigna unguiculata* (L.) Walp]

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Abstract

The optimum conditions for the preparation of protein concentrate from cowpea were determined using response surface methodology. A central composite rotatable design, consisting of eight experimental points and five replications at the centre point, was used to investigate the effects of two independent variables, namely pH and NaCl concentration on four responses: protein content (%,), protein yield (%), reactive lysine (g/16 g N) and zinc content (mg/100 g). A second-order polynomial model was used for predicting the responses. Regression analysis indicated that more than 80% of the variation was explained by the fitted models. Experimental results showed that under optimum conditions (pH and NaCl concentration of 9.91 and 0.15 M, respectively) the protein content was $\geqslant$84%, protein yield $\geqslant$87%, reactive lysine $\geqslant$1.175 g/16 g N and zinc content $\geqslant$7.75 mg/100 g. These results were in agreement with those predicted, hence indicating the suitability of the model used.

Keywords: Cowpea; Protein concentrate; Optimisation; Response surface methodology; Central composite rotatable design

1. Introduction

Cowpea [*Vigna unguiculata* (L.) Walp] is a widely grown legume food crop of the tropics, used in the diets of humans and other mammals. Cowpea seeds are an excellent source of carbohydrate (50–60%) and an important source of protein (18–35%). They also contain an appreciable quantity of micronutrients such as vitamin A, iron and calcium (Borget, 1989; Prinyawiwatkul, McWatters, Beuchat, & Phillips, 1996). Like other grain legumes, incorporation of cowpea seed flour into most foods to increase their protein level, is limited by the presence of restricting antinutritional factors such as phytic acid, protease inhibitors and flatulence-causing sugars. Polyphenolic compounds are also found. They can interact with proteins and reduce their digestibility, as well as alter organoleptic and functional properties of the seed flour (Cheftel, Cuq, & Lorient, 1985; Mwasaru, Muhammad, Bakar, & Che Man, 1999a; Okafor, Abara, Nwabuko, & Ogbanna, 2002). Polyphenolic compounds also have beneficial effects due to their antioxidant activity which is fundamental to life process (Rice-Evans, Miller, & Paganga, 1997). However, in practice, the elimination or reduction of these antinutrients is important to improve the utilization of grain legume seeds for food. Attention is now focused on the use of protein concentrates rather than the flour of legume seeds, since they have superior functional properties and are free of toxic factors and indigestible carbohydrates (Neto, Narain, Silva, & Bora, 2001).

In this connection, several methods have been reported in the literature for protein extraction and protein concentrates preparation from grain legumes (Jyothirmayi, Prabhakara, & Walde, 2006; Massoura, Vereijken, Kolster, & Derksen, 1998; Mizubuti, Biondo, Souza, da Silva, & Ida, 2000; Moure, Sineiro, & Dominguez, 2001; Quanhong & Caili, 2005). They involve the variation of physicochemical parameters such as pH, ionic strength, temperature,

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extraction time and solid/liquid ratio to give different concentrate and protein yields. According to these studies, the temperature of 35 °C was found to be most suitable. In our optimisation experiments, pH and NaCl concentration were considered as the sole variables because they were found to be the most influential parameters for protein extraction from our preliminary studies involving other factors namely, extraction time, particle size and solid/liquid ratio.

The variation and combination of the two parameters for the best protein extraction could be obtained using response surface methodology (RSM). This is a combination of statistical and mathematical techniques that has been successfully used for developing, improving and optimising processes (Myers & Montgomery, 2002). It usually uses an experimental design such as a central composite rotatable design (CCRD) to fit a first or second-order polynomial by a least significance technique. Contour plots generated could be usefully employed to study the response surfaces and locate the optimum (Rastogi & Rashmi, 1999).

While the functional, physicochemical properties and nutritive quality of protein isolate from cowpea seeds have been investigated (Mwasaru et al., 1991a; Mwasaru, Muhammad, Bakar, & Che Man, 1999b, 2000; Rangel et al., 2004), there has been no reports on the optimisation of the protein concentrate preparation from cowpea. The purpose of the present work was to apply RSM to study the effect of protein extraction parameters such as pH and NaCl concentration on protein content, protein yield; reactive lysine and zinc content from cowpea, and to determine the optimum preparation conditions.

2. Materials and methods

2.1. Sample preparation

Seeds of the white variety of cowpea purchased from Mokolo market (Yaounde, Cameroon) were hand-picked, washed and rinsed in deionised water. They were soaked for 1 h in water at room temperature (25 ± 2 °C), dehulled and dried in an air convection oven at 50 °C for 72 h. The dried seeds were ground into fine flour, passed through a sieve of 150 μm mesh size, and stored in air-tight polyethylene bags at room temperature during analysis (3 weeks). The composition of flour was: moisture (5.77%), ash (4.67%), total lipids (1.21%) determined according to AOAC (1990), polyphenolics (0.12%) according to Singleton and Rossi (1965) as gallic acid equivalents after their extraction in 70% (v/v) aqueous acetone (Shahidi & Naczk, 1989), ethanol-soluble sugars (9.41%) as described by Montreuil, Spik, and Tollier (1981) after their extraction in hot 80% (v/v) aqueous ethanol (Cerning & Guilhot, 1973), protein (20.33%), reactive lysine (1.00 g/16 g N) and zinc (9.93 mg/100 g) contents were determined as explained in Section 2.4.

2.2. Experimental design

A central composite rotatable design (Sado & Sado, 1991) with two variables was used to study the response pattern and to determine the optimum combination of the variables. This design enables the uniformity of the magnitude of prediction error for all points at the same radial distance from the centre point. As this is the desirable way of locating the optimum point within the unknown region of interest (Myers & Montgomery, 2002). The variables optimised were NaCl concentration (0–0.5 M) and pH (7–11), each at five coded levels −1.41, −1, 0, 1 and 1.41 as shown in Table 1. The level of intervals selected was based on studies of Moure et al. (2001) and Mwasaru et al., 1999a. The CCRD shown in Table 2 was arranged to allow for fitting an appropriate regression model using a multiple regression program. Five replicates (treatments 9–13) at the centre of the design were used to allow the estimation of the pure error sum of squares. Experiments were randomised in order to minimise the effects of unexplained variability in the observed responses due to extraneous factors.

The variables were coded according to the following equation:

$$X_i = (x_i - \bar{x}_i)/\Delta x_i \quad (1)$$

where X_i is the coded value of an independent variable, x_i is the real value of an independent variable, $\bar{x}_i$ is the real value of an independent variable at the centre point, Δx_i is the step change. The specific codes are

$$X_1(\mathrm{pH}) = (x_1 - 9)/1.418 \quad (2)$$

$$X_2(\mathrm{NaCl}) = (x_2 - 0.25)/0.177 \quad (3)$$

2.3. Preparation of cowpea protein concentrate

The experiments were carried out in a random order of that presented for the CCRD (Table 2), using 10 g of cowpea flour mixed with 100 ml NaCl solution at each of the

Table 1
Experimental values and coded levels of the independent variables for central composite rotatable design (CCRD)

Variable	Symbols		Levels				
	Coded	Uncoded	−1.41	−1	0	1	1.41
pH	X_1	x_1	7.000	7.582	9.000	10.418	11.000
NaCl conc. (M)	X_2	x_2	0.000	0.073	0.250	0.427	0.500

Table 2
Central composite rotatable design arrangement and responses in terms of protein content, protein yield, reactive lysine and zinc content of protein concentrate

Exp. no.	pH	NaCl conc. (M)	Protein (%)	Yield (%)	Lysine (g/16 g N)	Zn (mg/100 g)
	(X_1)	(X_2)	(Y_1)	(Y_2)	(Y_3)	(Y_4)
1	−1	−1	84.44	79.45	1.12	11.55
2	1	−1	80.24	91.42	1.36	8.86
3	−1	1	75.98	82.93	1.42	7.50
4	1	1	73.04	94.47	1.45	9.57
5	−1.41	0	81.02	79.56	1.08	10.84
6	1.41	0	75.82	91.56	1.47	8.73
7	0	−1.41	83.96	85.98	10.3	8.96
8	0	1.41	76.64	92.21	1.30	8.00
9	0	0	84.48	85.64	1.12	7.82
10	0	0	83.72	90.22	1.18	7.93
11	0	0	85.66	86.44	1.21	8.72
12	0	0	84.34	88.46	1.25	6.93
13	0	0	84.22	84.01	1.23	7.17

indicated concentrations in the design. The mixture was stirred for 2 h at 35 °C and the pH was adjusted in the same manner using a Hanna Model HI 8521 pH-meter (Hanna Instruments, Portugal). The slurry was then stirred for 30 min at 4 °C, and separated at 2000*g* for 30 min using a Jouan Model GR 4.11 centrifuge (Jouan, Saint Nazaire, 44600, France). The pellet was dissolved in 100 ml of the initial NaCl solution while stirring, the pH adjusted to the initial value, the slurry was stirred for 30 min at 4 °C and then centrifuged as previously explained. The resulting supernatants of the two alkaline extractions were combined. After addition of 100 ml of 95% (v/v) ethanol, the mixture was adjusted to pH 4.5 and the precipitated proteins were filtrated under vacuum using a Whatman No. 1 filter paper. The protein concentrate was dried at 50 °C for 48 h, ground and passed through a 150 μm sieve. The protein and zinc contents, protein yield as well as reactive lysine were determined, as explained below.

2.4. Determination of dependent variables

2.4.1. Protein content

Protein content (%) was calculated by difference between crude protein (N × 6.25) and non-protein nitrogen. Crude protein was determined by Kjeldahl procedure (AOAC, 1990). Non-protein nitrogen was determined by the method of Bhatty and Finlayson (1973) as modified by Naczk, Diosady, and Rubin (1985) by which proteins were precipitated with 10% trichloroacetic acid (TCA) solution, and the resultant soluble non-protein nitrogen was determined according to the Kjeldahl procedure.

2.4.2. Protein yield

Protein yield (%) was estimated as the percentage of protein mass of the concentrate obtained, with respect to the initial flour protein mass. All the masses were estimated on a dry weight basis, and moisture content was determined according to AOAC (1990).

2.4.3. Reactive lysine

Reactive lysine (g/16 g N) was determined by dye binding procedure using 1-phenylazo-2-naphtol-6-sulfonic acid (Orange 12), as described by Hurrell, Lerman, and Carpenter (1979). A sample containing 15 mg 'Arg + His + Lys' was mixed with 4 ml half saturated sodium acetate, and 40 ml of Orange 12 reagent were added directly for 'Arg + His + Lys' determination; or after propionylation of lysine with propionic anhydride for 'Arg + His' determination. Difference in absorbance at 475 nm after 2 h reaction at ambient temperature in the dark was used for calculating reactive lysine. Absorbance measurements were performed using a Spectronic Model 601 spectrophotometer (Milton Roy Company, Rochester, NY 14625, USA).

2.4.4. Zinc content

Zinc content (mg/100 g) was determined by atomic absorption spectrophotometry using a Unicam Model 969 atomic absorption spectrophotometer (Unicam Limited, York Street, Cambridge, CB1 2PX, United Kingdom), after digestion of 0.25 g sample with 6 ml of concentrated nitric acid at 150 ± 5 °C for 6 h according to Laurent (1981).

2.5. Statistical analysis

A second-order polynomial equation was used to fit experimental data given in Table 2. The model proposed for the response is given as follows:

$$Y_i = a_0 + a_1X_1 + a_2X_2 + a_{11}X_1^2 + a_{22}X_2^2 + a_{12}X_1X_2 \quad (4)$$

where Y_i ($i = 1$–4) is predicted response for protein content, protein yield, reactive lysine and zinc content of protein concentrate. The a_0 value is that of the fitted response at the centre point of the design, a_1 and a_2 are linear terms, a_{12} is interaction effect, a_{11} and a_{22} are squared effects. Optimisation of the fitted polynomials was carried out using a graphical technique (Rastogi & Rashmi, 1999). The optimum condition was verified by conducting experiments

under that condition. Experimental and predicted responses were compared. The fitted polynomial equation was expressed as surface and contour plots. The computer software used for this study was STATISTICA (version 5.5, 2002; Statsoft Inc., USA).

3. Results and discussion

3.1. Fitting the models

Four responses, namely protein content (Y_1), protein yield (Y_2), reactive lysine (Y_3) and zinc content (Y_4), were measured during the preparation of protein concentrate from cowpea under conditions of variable pH and NaCl concentration. Multiple regression equations were generated relating the predicted responses Y_1, Y_2, Y_3 and Y_4 to coded levels of the variables. The regression coefficients obtained are shown in Table 3. Analysis of plots of experimental and predicted values for the four responses indicated a good fit ($p < 0.05$; $R^2 > 0.85$).

When a model has been selected, analysis of variance is calculated to assess how well the model represents the data (Sado & Sado, 1991). The data obtained using ANOVA appropriate to the experimental design are presented in Table 4. It appears that the presented model significantly represents the data. The protein content (Y_1) is significant at $p < 0.001$, reactive lysine (Y_3) at $p < 0.01$, protein yield (Y_2) and zinc content (Y_4) at $p < 0.05$. Furthermore, the high values of the correlation coefficients (R^2) for the responses suggest that the model is a good fit. On the other hand, the lack of fit for all fitted models was found to be insignificant ($p > 0.05$). The lack of fit measures the failure of the model to represent data in the experimental domain at points which are not included in the regression (Myers & Montgomery, 2002). From the above, it can be concluded that the selected model adequately represent the data for all responses so obtained.

Table 3
Estimated coefficients of the fitted second-order polynomial for different responses and their signification based on *t*-statistic

Coefficient	Protein	Yield	Lysine	Zn
a_0	84.486[a]	86.955[a]	1.198[a]	7.713[a]
a_1	−1.814[b]	5.069[a]	0.103[b]	−0.451
a_2	−3.257[a]	1.920[c]	0.097[c]	−0.588[c]
a_{11}	−3.279[a]	−0.767	0.068	1.100[b]
a_{22}	−2.333[a]	1.011	0.013	0.443
a_{12}	0.315	−0.107	−0.052	1.190[c]

[a] Significant at 0.1% ($p < 0.001$).
[b] Significant at 1.0% ($p < 0.01$).
[c] Significant at 5.0% ($p < 0.05$).

Table 4
Analysis of variance (ANOVA) for the fitted models and lack of fit

	df	Sum of squares			
		Protein	Yield	Lysine	Zn
Model	5	211.141[a]	247.060[c]	0.202[b]	19.029[c]
Lack of fit	3	5.103[d]	6.047[d]	0.037[d]	1.294[d]
Pure error	4	2.057	23.593	0.010	1.980
Total	12	230.130	275.129	0.250	23.014
R^2		0.967	0.893	0.809	0.853

[a] Significant at 0.1% ($p < 0.001$).
[b] Significant at 1.0% ($p < 0.01$).
[c] Significant at 5.0% ($p < 0.05$).
[d] Non-significant.

3.2. Response surface plotting

The response surfaces obtained from the determined coefficients (Table 3) are represented in Fig. 1a–d.

3.2.1. Effect of pH and NaCl concentration on protein content

Protein content of the cowpea concentrate is both a linear and quadratic function of pH and NaCl concentration. All the observed effects were significant at $p < 0.05$ (Table 3). It can be suggested from Fig. 1a that increase of pH and NaCl concentration during the preparation of protein concentrate from cowpea favours the extraction of non-protein compounds such as lipids and carbohydrates. These results agree with those of Mwasaru et al. (1999a). The predicted protein content optimum corresponding to 85.94% was reached at NaCl concentration coded of −0.72 (0.12 M) and at a pH coded of −0.31 (8.56). These coded values were obtained by resolution of the polynomial equation (4) using the partial derivative method described by Quanhong and Caili (2005), after substitution for protein coefficients given in Table 3.

3.2.2. Effect of pH and NaCl concentration on protein yield

Fig. 1b shows that increase of pH and NaCl concentration leads to an increase of protein yield, although at a faster rate with pH than with NaCl concentration. Linear effects of pH and NaCl concentration were found to be positive and significant at $p < 0.001$ and $p < 0.05$, respectively (Table 3). This increase of protein extractability was also observed by Mwasaru et al. (1999a) for cowpea, Moure et al. (2001) for dog rose (*Rosa rubiginosa*) and Jyothirmayi et al. (2006) for *Erythrina variegata* seeds.

3.2.3. Effect of pH and NaCl concentration on reactive lysine

The content of reactive lysine of cowpea protein concentrate increased linearly with increase of pH and NaCl concentration, although at a faster rate with pH than with NaCl concentration (Fig. 1c). These effects are positive and significant at $p < 0.01$ and $p < 0.05$, respectively, for pH and NaCl concentration (Table 3). These observations indicate an increasing elimination with the pH or NaCl concentration of antinutritional compounds such as flatulence-causing sugars and polyphenolics which interact with the lysine residues of proteins, thus reducing their availability (Besançon, 1999; Cheftel et al., 1985).

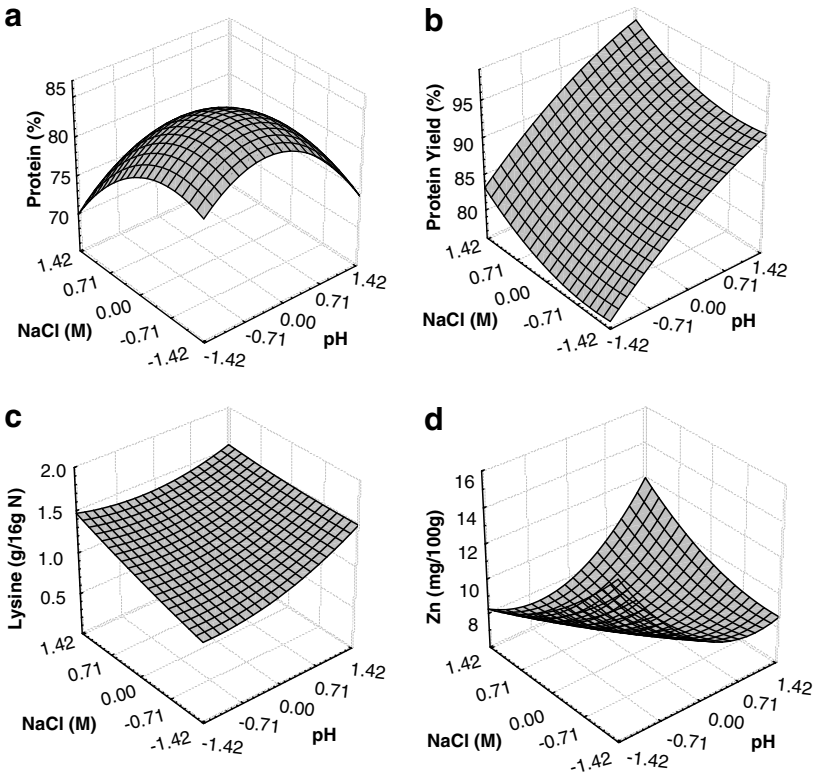

Fig. 1. (a–d) Response surfaces showing effect of pH and NaCl concentration on (a) protein content (b) protein yield (c) reactive lysine and (d) zinc content.

3.2.4. Effect of pH and NaCl concentration on zinc content

Zinc content of cowpea protein concentrate linearly depended on NaCl concentration, and quadratically on pH. The linear effect was found to be negative and significant at $p < 0.05$, while the quadratic effect was found to be positive and significant at $p < 0.01$. The interaction between these two variables was significant at $p < 0.05$ (Table 3). At higher coded values of pH and NaCl concentration, zinc content increased with an increase in pH and NaCl concentration, while at lower coded values of pH and NaCl concentration, zinc content decreased with increase of pH and NaCl concentration (Fig. 1d). It could be concluded that at low or high levels of NaCl concentration and pH, extraction and recovery of zinc linked-proteins are favoured.

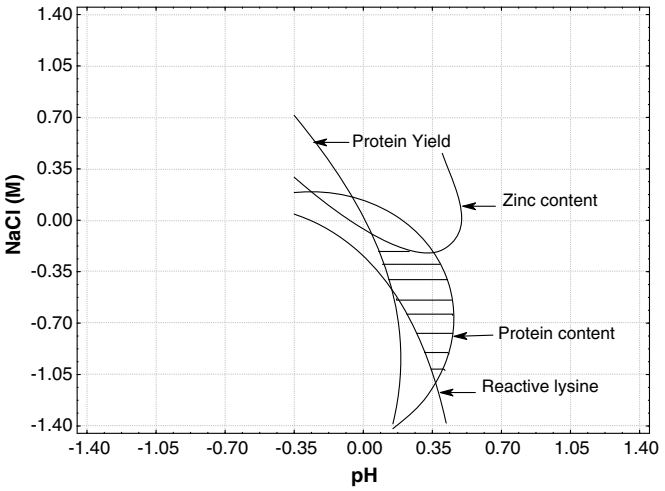

Fig. 2. Superimposed contour plots showing the shaded overlapping area.

3.3. Optimisation

From the above results, it is clear that the optimum conditions to obtain protein concentrate from cowpea vary for each of the four responses studied, namely protein content (Y_1), protein yield (Y_2), reactive lysine (Y_3) and zinc content (Y_4). An acceptable compromise was made based on the following criteria: $Y_1 \geqslant 84\%$, $Y_2 \geqslant 87\%$, $Y_3 \geqslant 1.175$ g/16 g N and $Y_4 \geqslant 7.75$ mg/100 g. These specifications also served as constraints on optimisation, and the graphic optimisation technique by superimposition of the three-dimensional response surfaces was adopted (Rastogi & Rashmi, 1999). The contour plots for each of the responses generated were superimposed, and a combination of the regions that best satisfied all the constraints was selected as the optimum conditions and represented by the shaded area (Fig. 2). The point at pH 9.91 and NaCl concentration = 0.15 M of this area was selected for experimental verification of the results.

Table 5
Optimum condition, predicted and experimental value of response at optimum condition

Optimum condition	Coded levels	Actual levels	
pH	0.64	9.91	
NaCl concentration	−0.54	0.15	
Responses	Predicted value	Experimental value[a]	
		Mean	Range
Protein content (%)	84.01	84.19 ± 1.08	83.11–85.27
Protein yield (%)	88.36	86.90 ± 2.07	83.59–89.00
Reactive lysine (g/16 g N)	1.22	1.29 ± 0.07	1.21–1.36
Zn content (mg/100 g)	7.90	8.28 ± 0.31	7.88–8.65

[a] Mean of four determinations.

3.4. Verification of results

The suitability of the model equation for predicting the optimum response values was tested using the optimum conditions selected. This set of conditions were determined to be the optimum by the response surface methodology approach and were also used to validate experimentally and predict the values of the responses using model equations. The experimental values were found to be in agreement with the predicted ones (Table 5).

4. Conclusions

The response surface methodology is an important tool that allows following of the evolution and the optimisation of processes from an appropriate experimental design of a limited number of experiments. The preparation of protein concentrate from cowpea flour is significantly influenced by the pH and the NaCl concentration. Linear and quadratic effects of these two variables affect protein content, protein yield, reactive lysine and zinc contents of the cowpea protein concentrate. A significant interaction existed between pH and NaCl concentration for zinc content. The optimum conditions predicted, after superimposition of the contour plots verifying the constraints values were found to be in agreement with the experimental results.

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